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Cytokine & Growth Factor Reviews xxx (2005) xxx–xxx
Cytokine & Growth Factor Reviews
www.elsevier.com/locate/cytogfr

Cytokines and junction restructuring during spermatogenesis—a lesson to learn from the testis

Weiliang Xia, Dolores D. Mruk, Will M. Lee, C. Yan Cheng *

Population Council, Center for Biomedical Research, 1230 York Avenue, New York, NY 10021, USA

Abstract

In the mammalian testis, preleptotene and leptotene spermatocytes residing in the basal compartment of the seminiferous epithelium must traverse the blood-testis barrier (BTB) during spermatogenesis, entering the adluminal compartment for further development. However, until recently the regulatory mechanisms that regulate BTB dynamics remained largely unknown. We provide a critical review regarding the significance of cytokines in regulating the ‘opening’ and ‘closing’ of the BTB. We also discuss how cytokines may be working in concert with adaptors that selectively govern the downstream signaling pathways. This process, in turn, regulates the dynamics of either Sertoli–Sertoli tight junction (TJ), Sertoli–germ cell adherens junction (AJ), or both junction types in the epithelium, thereby permitting TJ opening without compromising AJs, and vice versa. We also discuss how adaptors alter their protein–protein association with the integral membrane proteins at the cell–cell interface via changes in their phosphorylation status, thereby altering adhesion function at AJ. These findings illustrate that the testis is a novel in vivo model to study the biology of junction restructuring. Furthermore, a molecular model is presented regarding how cytokines selectively regulate TJ/AJ restructuring in the epithelium during spermatogenesis.

Keywords: Spermatogenesis; Testis; Junction restructuring; Cytokines; TGF-β3; TNFα; p38 MAPK; ERK; JNK; Blood-testis barrier; Adherens junction; Tight junction; Adaptors; Ectoplasmic specialization

1. Introduction

The production of mature spermatozoa (haploid, 1*n*) from spermatogonia (diploid, 2*n*) is essential for the perpetuation of all mammalian species. Such event, known as spermatogenesis in the male, takes places in the functional unit of the testis called the seminiferous tubule. Seminiferous tubules, in turn, coordinate with Leydig cells in the interstitium and the brain via the hypothalamic-pituitary-testicular axis to regulate spermatogenesis [1,2]. Although spermatogenesis varies in detail in different species (e.g., minks are seasonal breeders exhibiting seasonally or environmentally responsive phases in this process whereas spermatogenesis continues throughout the entire life span in humans and rodents), the cellular constituents and the basic physiology of the testes are rather similar [3]. We limit our discussion largely in rats, mice and/or men since most studies were conducted in these species.

Spermatogenesis can be divided into three distinct phases which provide an upward of 150×10^6 spermatozoa per day per man [1,3]. The germline stem cells spermatogonia can either self-proliferate (phase 1) or differentiate into primary spermatocytes, which then undergo meiosis and differentiate into secondary spermatocytes and eventually haploid spermatids (phase 2). These cells, in turn, differentiate morphologically and functionally to spermatozoa via spermiogenesis (phase 3), which are released into the tubule lumen at spermiation [1,3]. This entire process of germ cell development in the seminiferous epithelium is dependent on temporal and spatial expression of unique sets of genes and proteins. In the rat testis, an epithelial cycle (∼12–14 days duration) can be divided into 14 stages which are classified according to the unique germ cell types that associate with Sertoli cells in the epithelium [3,4]. It takes ∼58 days for a single spermatogonium to fully differentiate and develop into 256 spermatozoa. As such, it takes ∼4.5 epithelial cycles for one spermatogonium to differentiate into 256 spermatids. For each stage, at least four germ cell types are present in the epithelium that are organized

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* Corresponding author. Tel.: +1 212 327 8738; fax: +1 212 327 8733.
E-mail address: Y-Cheng@popcbr.rockefeller.edu (C.Y. Cheng).

1359-6101/$ – see front matter © 2005 Published by Elsevier Ltd.
doi:10.1016/j.cytogfr.2005.05.007

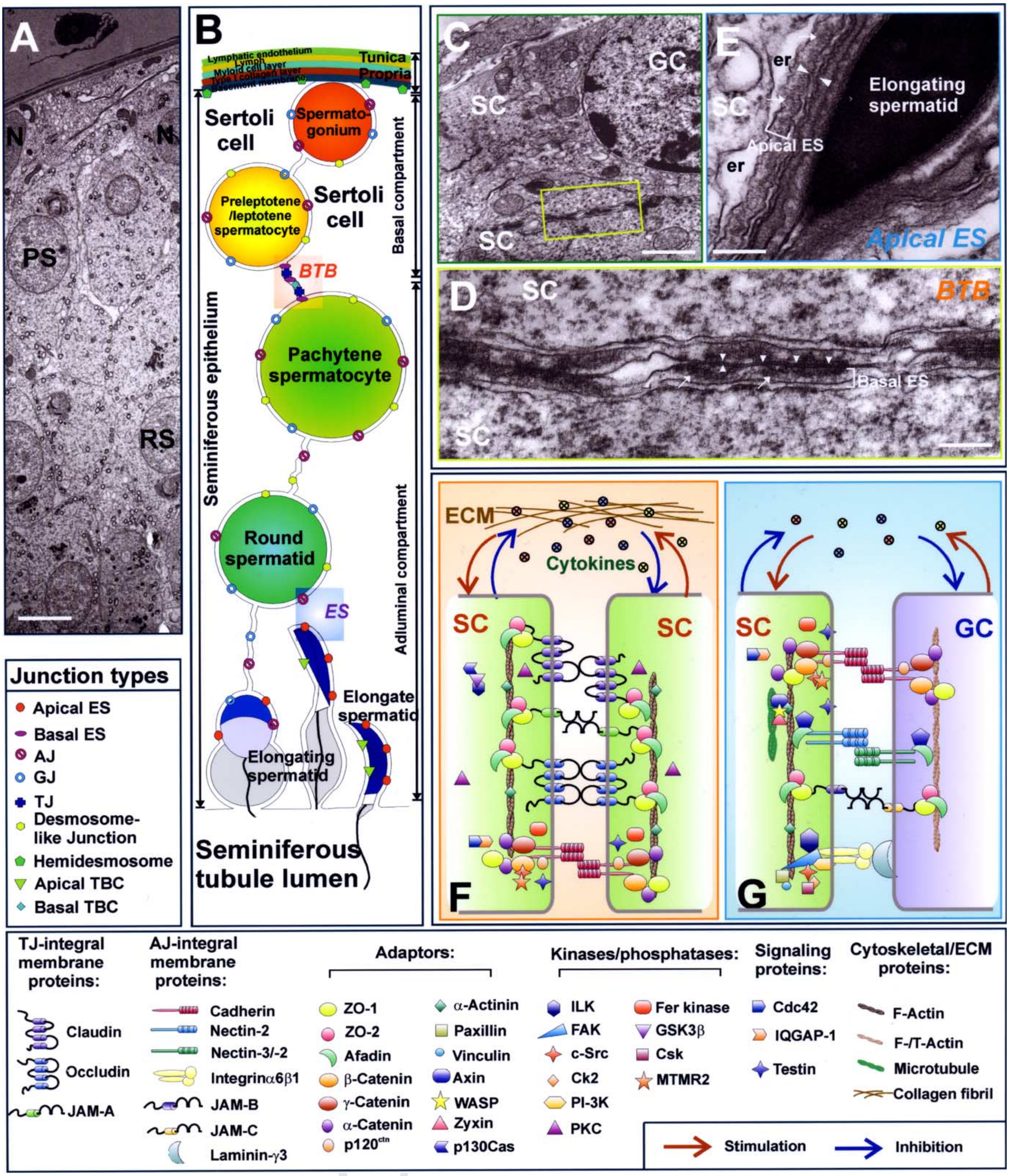

Fig. 1. Spermatogenesis and cell junctions in the seminiferous epithelium of the mammalian testis (e.g. rats). (A) This is the cross-section of a seminiferous tubule from an adult rat testis showing the intimate relationship between Sertoli cells (N, Sertoli cell nucleus) and germ cells (e.g., pachytene spermatocyte PS, round spermatid RS). (B) Schematic drawing of developing germ cells and their intimate relationship with Sertoli cells during spermatogenesis in the seminiferous epithelium. Also shown is the relative location of different junction types in the epithelium between Sertoli cells as well as between Sertoli and germ cells. Sertoli and germ cells constitue the seminiferous epithelium that is adjacent to the tunica propria. Differentiating germ cells must migrate from the basal to the adluminal compartment, traversing the BTB, which has physically divided the epithelium into the basal and adluminal compartment. (C–E) Electron micrographs of cross sections of seminiferous epithelium illustrating the ultrastructural features of the blood-testis barrier at low (C) and high (D) magnification. The basal ES is characterized by the presence of actin filament bundles (white arrows) sandwiched between the cisternae of endoplasmic reticulum (er), and the

spatially into layers from the base to the lumen of the seminiferous tubule [3,4]. Furthermore, spermatogenesis cannot complete without the support of Sertoli cells, which are the only other cell type in the seminiferous epithelium behind the BTB besides germ cells (note: the BTB has physically divided the epithelium into the basal and adluminal compartment, see Fig. 1) [5–7]. Except for the spermatogonia, developing germ cells move progressively toward the lumen [8]. For instance, preleptotene and leptotene spermatocytes that lie at the periphery of the tubule and outside the BTB must traverse the BTB at late stages VIII and early IX of the epithelial cycle [8].

It is conceivable that enormous Sertoli–germ cell interactions take place in the seminiferous epithelium throughout spermatogenesis [1–4,6,9,10]. If one views spermatogenesis as a voyage of a germ cell that moves from the basal to the adluminal compartment while developing to a mature spermatozoon, this process involves numerous decision makings and executions. It also requires signalings in and out of germ cells to facilitate this event. Although it is not entirely clear regarding the sequence of these signals, there are at least two sources: external signals from outside the tubule (e.g., via Leydig cells, peritubular myoid cells, and both paracrine and hormonal factors including those from the pituitary gland), and internal crosstalks between germ and Sertoli cells (e.g., integrin-mediated signalings) [5,6,11,12]. The phenotypic consequence of these signalings is manifested, at least in part, via the constant remodeling at the Sertoli–Sertoli and Sertoli–germ cell interface where different cell junction types are present [1,2].

The identities of these signals and the details of the remodeling events have become increasingly clear in recent years [1,2]. For instance, there is accumulating evidence that illustrates the crucial roles of cytokines pertinent to spermatogenesis and junction restructuring [2]. In this review, we first give an update on the junction complexes that are found in the testis, highlighting how cytokines (e.g., TGF-β3, TNFα) can affect junction dynamics and how these signals are being fine-tuned to allow their regulation of a particular junction type.

2. The seminiferous epithelium: Sertoli–germ cell junctions and spermatogenesis

2.1. Seminiferous epithelium

The seminiferous epithelium is composed of Sertoli and germ cells. The Sertoli cell is by and large a tall columnar cell extending from the base to the apex of the seminiferous tubule [3]. It is physically reshaped by germ cells to possess many cytoplasic processes because each Sertoli cell is ‘nursing’ about 30–50 germ cells at different stages of their development at any given time during the epithelial cycle [13,14]. In the rat, Sertoli cells cease to proliferate at about day 20 postnatal and the number of these nursing cells determines how many germ cells can be supported and produced via spermatogenesis in the testis [3], illustrating the crucial function of Sertoli cells. For instance, Sertoli cells provide structural support for germ cells and their translocation, create the BTB and define the polarity of the epithelium, secrete numerous biological factors and nutrients for germ cells, and conduct other vital functions pertinent to spermatogenesis (e.g., phagocytosis) [2,3,14].

2.2. Sertoli–Sertoli and Sertoli–germ cell junctions

The different junction types that are found in the seminiferous epithelium have recently been reviewed [1,2]. Similar to other epithelia or endothelia, virtually all major junction types are found in the testis. Besides the tight junctions that are restricted to the BTB, several anchoring junction types (four are found in most epithelia) are also detected in the testis: (a) adherens junction (including basal and apical ectoplasmic specialization [ES], basal and apical tubulobulbar complex [TBC]); (b) desmosome-like junctions; and (c) hemidesmosomes (for reviews, see [1,2,10,15]). ES is a testis-specific, actin-based adherens junction localized at two sites in the seminiferous epithelium: basal and apical compartment (see Fig. 1) [2,9,10,16,17]. Basal ES is limited to BTB and present side-by-side with TJ (Fig. 1). Apical ES is found between elongating/elongate spermatids and Sertoli cells. At least three protein complexes, namely, the cadherin/catenin, the nectin/afadin, and the integrin/laminin, are known to be ES components [2,17]. TBC is another modified AJ type found in the testis [2,10,18]. Apical TBC only appears a few days before spermiation in the epithelium at late stage VIII of the epithelial cycle when apical ES begins to disappear whereas basal TBC co-exists with TJ, basal ES, and desmosomal-like junctions at the BTB site. Desmosome-like junctions are present between Sertoli cells and spermatogonia, spermatocytes and round spermatids, being most prominent surrounding pachytene spermatocytes [10]. The BTB is not fully formed until 16–19 days postnatal in the rat testis [3]. Unlike barriers in other organs (e.g., the blood–brain barrier, the blood–retinal barrier) where TJs are localized to the apical region of the epithelium/endothelium, to be

Sertoli cell membrane (apposing arrowheads represent the apposing Sertoli cell membranes), which can be found on both sides of the apposing Sertoli cells. Tight junction (TJ) is found between the basal ES, the coexisting TJ and basal ES in turn constitute the BTB. Apical ES is shown in (E) which is typified by the presence of actin filament bundles (white arrowheads) sandwiches between the cisternae of er and Sertoli cell membrane (apposing white arrowheads represent the apposing Sertoli and germ cell membranes). However, this typical feature of ES, in contrast to the basal ES, is restictied only to the Sertoli cell side in apical ES. (F–G) Schematic drawings that illustrate the molecular architecture of the constituent proteins at the BTB (F) and apical ES (G), which include cytokines (e.g., TGF-β3 and TNFα) released from Sertoli and/or germ cells can mediate Sertoli–germ cell crosstalk during spermatogenesis. The protein complexes known to exist at the apical ES site include cadherin/catenin, nectin/afadin, and α6β1 integrin/laminin γ3; whereas occludin/ZO-1, JAM/ZO-1, claudin/ZO-1, cadherin/catenin and nectin/afadin are found at the BTB site. Bar in E = 10 μm, C = 3 μm, D = 0.25 μm and E = 0.3 μm, respectively.

followed by AJ, and TJs are furthest away from the ECM; TJs at the BTB lie closest to the basement membrane (a modified form of ECM). Furthermore, BTB is a dynamic structure which must ‘open’ and ‘close’ to permit preleptotene/leptotene spermatocyte transmigration. BTB is a rather complex barrier when compared to other barriers (e.g., gastric–mucosal barrier which is formed by epithelial cells, blood–retinal barrier and blood–brain barrier which are formed by endothelial cells) [19–21] (see Fig. 1). Recent studies have also shown that apical ES is constituted and regulated by proteins that are usually restricted to the focal contact in cell–matrix interface in other epithelia [22]. This hybrid cell–matrix–cell junction type may indeed be essential for rapid junction remodeling to facilitate spermatids orientation and movement at spermiation.

2.3. Constituent proteins of different junction types in the testis

2.3.1. Tight junction (TJ)

TJ is the only known example of occluding junction that confers the barrier function of an epithelium or endothelium by restricting the passages of molecules through the intercellular spaces and creates a boundary that defines cell polarity [23]. In the testis, TJ also creates an immunological barrier that sequesters the post-meiotic germ cell antigens from the immune system of the host animals. The currently known TJ integral membrane proteins include JAMs (junctional adhesion molecules), claudins and occludins, which have recently been reviewed [1,2,23,24], as such, only a brief update is provided in this section.

2.3.1.1. JAMs. JAMs are members of a distinct class of cell adhesion molecules typified by the presence of two Ig-like loops in the extracellular domain that are expressed in leukocytes and are localized to tight junctions as integral membrane proteins in epithelial and endothelial cells [25,26]. Since the discovery of JAM-A in 1998 [27], other members, including the more related JAM-B and JAM-C, and the less related JAM4, coxsackie and adenovirus receptor (CAR), and endothelial cell-selective adhesion molecule (ESAM), have recently been added to the list [25,26,28]. The presence of JAM-A, B and C in the testis have now been confirmed [29,30]. JAM-A is present at the BTB in the rat testis, co-localizing with ZO-1 [30]. Moreover, JAM-A expression is stage-specific, being highest at IX–XIV, lowest at IV–VI [30]. This stage specificity apparently is related to its possible involvement in BTB dynamics, facilitating the passage of preleptotene/leptotene spermatocytes across the BTB. Although Jam-*A*$^{-/-}$ mice has been generated, it is not known if the BTB is affected since a morphological examination of the testis has yet to be reported [31]. A recent study on the *Jam-C*$^{-/-}$ mice have shown that JAM-C is crucial to spermiogenesis since in the viable mutants, mature spermatids are missing [29]. In normal mice, JAM-C is localized to the developing round and elongating spermatids [29]. Interestingly, JAM-B has been localized both to the site of TJs at the basal compartment and to the apical ES at the spermatid-Sertoli cell interface in the seminiferous epithelium, outside the BTB [29]. Besides their homophilic interactions amongst JAM-A, B and C, JAM-C can interact with JAM-B heterotypically [32]. Both JAM-B and JAM-C are localized to the heads of spermatids at the apical ES, and this heterophilic association may be important for the Sertoli cell-spermatid adhesion function [29]. Based on currently available data, two roles are suggested for JAMs: in the immune system they are crucial to leukocyte transmigration; and in polarized epithelial and endothelial cells, they seem to take part in organizing TJ and cell polarity [26]. This latter physiological role has been extended to the testis since the cell polarity complex [partitioning-defective (Par) 3/atypical protein kinase C (aPKC)/Cdc42] apparently is recruited by JAM-C to facilitate round spermatid polarization and thus differentiation [26]. How JAMs assist preleptotene/leptotene spermatocytes to traverse the BTB similar to neutrophil transmigration across the endothelial TJ-barrier remains to be investigated since germ cells per se, unlike neutrophils or macrophages, are not actively migrating cells. It is possible that JAMs are associated with other motor proteins (e.g., myosin VIIa) and cytoskeletons (e.g., actin, tubulin) that facilitate germ cell movement using the locomotive apparatus in Sertoli cells that provides the necessary protrusive force to guide germ cell movement (for review, see [2]).

JAMs are expressed in multiple epithelia, endothelia, leukocytes and platelets [25,26]. The regulation of JAMs in the testis is largely unknown. In the rat testis, when the intratesticular T was suppressed by placing testosterone and estrogen implants subdermally, spermatids (step 8 and beyond) were depleted because of a disruption of the cell adhesion at the ES [30,33–36]. However, the tight junctions at the BTB remained intact which were associated with a significant surge in the levels of JAM-A, occludin and ZO-1 in the epithelium [30]. Indeed, the JAM-A distribution at the BTB site in the basal compartment of the seminiferous epithelium was significantly induced and intensified, becoming a thickened and prominent ring surrounding the entire tubule [30]. It is apparent that a depletion of androgen in the testis triggers a novel mechanism that leads to two distinctive events: germ cell loss and a reinforced BTB [30]. Another model using Adjudin to induce germ cell sloughing from the epithelium in adult rat testes has yield similar results in which JAM-A expression was induced at the time of germ cell depletion (unpublished observations). Although the compounds that were used to trigger the changes in the epithelium are different in these two models, namely androgen suppression and Adjudin, the signaling events (e.g., both treatments activate the integrin/focal adhesion kinase signaling pathway) and the phenotypic outcome (e.g., germ cell loss from the epithelium and a reinforced BTB) are similar [36,37]. This seemingly suggests that JAM-A is regulated, at least in part, by a mechanism downstream of lowered intratesticular T level that triggers germ cell sloughing from the epithelium. It is not known if cytokines are the upstream regulators of JAMs. An earlier report has shown that TNF and IFN-γ treatment of human umbilical vein endothelial cells can reduce cell surface expression of JAM-A, but these cytokines have no effects on the rate of transmigration of neutrophils [38].

2.3.1.2. Claudins. The claudin superfamily of TJ integral membrane proteins consists of at least 24 members with Mr ranging between 20 and 27 kDa [39,40]. Claudins have a unique expression profile in a tissue [39]. For instance, claudin-1 and claudin-11 are expressed in the testis, mostly restricted to Sertoli cells, and the brain, whereas more than 10 claudin members are expressed in the kidney [39]. In the testis, the expression of claudin-3, -4, -5, -7, -8 has also been reported [2,41]. Claudin-11, also known as oligodendrocyte-specific protein (OSP), is the best studied claudin in the testis [42–46]. *Cld-11*$^{-/-}$ mice were sterile and were associated with the absence of TJ strands in the seminiferous epithelium and in the myelin sheath in the brain [46]. Claudin-11 is known to be up-regulated by androgens [42,47] and down-regulated by TGF-β3 [44] in Sertoli cells cultured in vitro. Claudin-11 expression is high from postnatal days 10–16 in the rat testis corresponding to the maturation of BTB [43]. Anti-androgen, such as flutamide, can also inhibit the expression of claudin-11 in prepubertal rat testes [42]. Claudin-11 is also important for hearing function since *Cld11*$^{-/-}$ mice lacking TJ in the basal cells of stria vascularis in cochlea failed to compartmentalize the endolymph and suppressed electrical potentials [48].

TJ strands in the intercellular junction are not a static but dynamic structure. A recent study by real-time imaging to examine the behavior of exogenously expressed claudin-1 in mouse L fibroblasts showed that the paired claudin strands underwent constant and dynamic reorganization while maintaining the structural integrity of the entire TJ network [49]. Internalization of claudin-3 was also observed via endocytosis in confluent epithelial cells, after it was dissociated from other TJ components, such as JAM, occludin and ZO-1 [50]. This dynamic nature of claudins, plausibly applicable to other TJ constituent proteins, is not entirely unexpected since TJ barriers must undergo conformational changes to accommodate paracellular transport of substances, such as during food adsorption in the small intestine. For the BTB, it has to be 'opened (or 'dissolved' ?) and then 'closed' (or 'regenerated' ?) frequently to facilitate germ cell passage while maintaining the barrier function during the epithelial cycle. It is likely that such reorganization of claudin strands, possibly also of occludin- and JAM-constituted TJ strands, are occurring at the BTB. The uncoupling of TJ proteins may indeed be a prerequisite for the dual roles played by the BTB during its restructuring to permit germ cell passage while maintaining the barrier function simultaneously.

2.3.1.3. Occludin. Occludin is the first TJ integral membrane protein found in epithelia [51] and is the most studied in this category. Occludin is known to be regulated by cytokines in the testis (e.g., TGF-β2, TGF-β3) [44,52]. Other signaling events are recently shown to engage in its regulation as well. In the androgen-suppressed rat testes to induce germ cell loss from the epithelium, occludin expression, similar to JAM-A, is significantly induced, resulting in prominent staining at the BTB when Sertoli–germ cell adhesion function was compromised [30]. This also reinforces the notion that the regulation of TJ proteins is essentially different from that of AJ proteins in the rat testis. Occludin is also regulated, at least in part, by ubiquitination [53]. Itch (an E3 ubiquitin ligase) and UBC4 (an ubiquitin-conjugating enzyme) are reciprocally regulated versus occludin during Sertoli cell TJ assembly or disassembly, and ubiquitin-conjugated and Itch-conjugated occludin are detected when the dibutyryl-cAMP-induced degradation of occludin is blocked by a proteasome inhibitor MG-132 [53]. Other cytokines, such as TNFα, IL-1β, IFN-γ, can also affect occludin expression and its distribution at TJs in multiple epithelia and endothelia [54–56]. It is not known if these cytokines can exert any effect on occludin expression or its cellular distribution at the BTB. But TNFα has been shown to perturb TJ-barrier function in Sertoli cell cultures [57] and can cause germ cell exfoliation in the rat testis after its systemic administration [58]. Recent studies have shown that TNF and IFN-γ can indeed regulate occludin transcription by diminishing its promoter activity [55].

2.3.2. Anchoring junction

2.3.2.1. The cadherin/catenin protein complex. Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell–cell adhesion in multiple epithelia including the seminiferous epithelium in the testis [59,60]. The cadherin superfamily consists of over 80 members that fall into at least six subfamilies, which include (i) the type I classical cadherins (e.g., E-cadherin, N-cadherin, P-cadherin) and its highly related; (ii) type II classical cadherins (e.g., VE-cadherin); (iii) desmosomal cadherins (desmocollins and desmogleins); (iv) protocadherins; (v) seven-pass transmembrane cadherins (Flamingo); and (vi) Fat-like cadherins [59–62]. Type I classical cadherins are the best studied cadherins in multiple tissues including the testis. Besides the classical cadherins, the presence of other subfamilies in the testis, such as protocadherins, Fat and Flamingo, has also been detected by RT-PCR [63], but their function in the seminiferous epithelium are less known. During development, the expression of different cadherins is highly dynamic [64] and this seems to be applicable to the testis as well since the expression profile of cadherins varies with the age and cell types in the rat testis where at least 24 cadherins are known to be present [63]. For instance, N-cadherin is predominantly localized to the basal ES and the periphery of the seminiferous tubules with restricted and stage-specific localization at the apical ES [30,65–69], whereas E-cadherin is relatively more abundant in germ cells [63,66]. A smaller amount of N-cadherin in the testis appears also to be a component of desmosomal-like junctions which is a hybrid junction type of desmosome and gap junctions [65,67]. Indeed, N-cadherin has been shown to link to both actin microfilament and microtubules in the testis [66]. A recent report has also illustrated that protocadherin α3 is associated with spermatids at the acrosomal area, intercellular bridge as well as flagellum, distinct from the distribution of classical cadherins [70].

Classical cadherin-based protein complex comprising of the transmembrane protein cadherins and intracellular adaptor catenins is a well defined focal point of cell adhesion and signaling [59,71]. β-Catenin and γ-catenin connects cadherins to α-catenin and α-actinin, which are two putative actin binding proteins [72]. Phosphorylation of β-catenin can in turn regulate the integrity of the cadherin/catenin complex [73]. In both Adjudin- and androgen suppression-induced germ cell loss models, the event of germ cell loss is facilitated by the dissociation of N-cadherin from β-catenin [30,35,68]. Indeed, increased tyrosine phosphorylation of β-catenin was detected at the time of germ cell depletion in these models [30]. Kinases and phosphatases are also known to regulate cadherin/catenin association [35,74,75]. For instance, myotubularin-related protein 2 (MTMR2), a lipid phosphoinositide phosphatase, was shown to interact with the kinase c-Src [35] and c-Src in turn associates with the N-cadherin/β-catenin complex [74]. This illustrates a novel regulatory mechanism may be in place in the testis regarding the cadherin/catenin-mediated cell adhesion function in which MTMR2 and c-Src regulate the phosphorylation status of the cadherin/catenin, which in turn determines its cell adhesive function. More recent studies have shown that the N-cadherin/β-catenin adhesion unit can also be regulated by the equilibrium between IQGAP-1 (IQ motif containing GTPase activating protein, an effector of Cdc42 GTPase) and Cdc42 in Sertoli–germ cell AJ [76]. For instance, using a Ca^{2+} switch model, it has been demonstrated that at low Ca^{2+} level, IQGAP-1 is released from Cdc42, and interacts with β-catenin instead, causing the dissociation of β-catenin from N-cadherin, and germ cell depletion from Sertoli cells [76].

E-Cadherin is also a tumor suppressor which is down-regulated while N-cadherin is up-regulated during epithelial tumor progression [64,77,78]. This ‘cadherin switch’ further illustrates the unique yet pivotal role of each cadherin in cell adhesion and cell motility. It is not clear if such dynamic switch-over between different cadherins occur during germ cell movement in the seminiferous epithelium. However, N-cadherin can become highly expressed in the testis of Adjudin treated rats during germ cell loss from the epithelium [66–68]. N-cadherin is also up-regulated in androgen suppressed rat testes during germ cell loss [30,35]. Yet such a surge in N-cadherin cannot rescue germ cell loss from the epithelium since a loss of association between N-cadherin and β-catenin was detected at the time of germ cell sloughing in both models [30,35]. It seems that such an induction of cadherins reinforces the BTB integrity since N-cadherin is also a component protein of the BTB in the rat testis.

2.3.2.2. The nectin/afadin/ponsin/ADIP complex. The nectin/afadin/ponsin complex is another actin-based cell adhesion protein complex that plays a crucial role in the testis during spermatogenesis. It confers Sertoli–germ cell adhesion function particularly for elongating/elongate spermatids [1,2,68,79]. Four nectins (nectin-1, -2,-3, and -4) have been identified thus far, all of which are expressed in the testis with nectin-2 and nectin-3 being the highly expressed [2,80–82]. Nectin-3 is restricted exclusively to elongating/elongate spermatids which can heterotypically interacting with nectin-2 on the Sertoli cell side [68,83]. Spermatozoa from *nectin-2*$^{-/-}$ mice were morphologically aberrant and functionally impotent [83–85]. Since nectins are capable of activating Cdc42 via c-Src and a Cdc42 GEF (GDP/GTP exchange factor) [86], or activating Rac, thus recruiting the polarity complex Par3/aPKC/Par6 to the apical ES site [87], the absence of nectin-3 may also lead to malfunctioning of spermatid polarization, similar to *Jam-C*$^{-/-}$ mice [29]. Nectins are known to initiate cell–cell contacts by recruiting cadherin and JAM-A to establish functional AJ and TJ in epithelial cells [79,87–89]. It is likely that nectin-2/-3 and JAM-B/-C can also interact with each other since they are all localized to the elongating/elongate spermatids at the apical ES site, which should be investigated in future studies. In the Adjudin-induced germ cell loss model, it was found that the nectin-3/afadin interaction became severely weakened before any obvious reduction in their protein levels was detected [68], illustrating this cell adhesion unit must be compromised to facilitate spermatid loss (Table 1).

Besides afadin and ponsin, cytoplasmic adaptors that link nectin to the actin-based cytoskeleton [79], a new adaptor protein ADIP (afadin DIL domain-interacting protein) has recently been localized to AJ sites that interacts with both α-actinin and afadin, providing additional cytoplasmic link between nectin- and cadherin-based cell adhesion units [90,91]. ADIP is highly expressed in the mouse testis [90]. Another possible linker that binds to both afadin and α-actinin is LMO7 (LIM domain only 7), however, its presence in the rat testis failed to be confirmed by immunoblot analysis [92].

Nectin-like (Necl) molecules are similar to nectins, but do not bind to afadins [87,88]. This group of calcium-independent cell adhesion molecules consists of five members, capable of homo- or heterophilic interactions with nectins, and are important cell–cell adhesion molecules in various tissues [87,88]. At least Necl2 has been shown to be highly expressed in the rat testis [93]. It will be important to explore the significance of Necls in the testis, which is likely to involve in Sertoli–germ cell adhesion function.

Table 1
Cytokine-mediated regulation of junction component proteins in epithelia including the testis

Junction component	Protein	Cytokine/hormone that modulates the steady-state mRNA/protein level (+/−) or protein distribution pattern (d) of the target junction protein	Selected references
TJ-integral membrane	JAM-A	TNF (d), IFN-γ (d), T↓(+)	[30,38]
	Occludin	TGF-β3 (−), HGF (−/d), TNF (−/d), IFN-γ (−/d), VEGF (−/d, inhibited by ANP), IL-1β (−/d), IL-4 (−), IL-13 (−), MCP-1 (−), T↓(+)	[44,52,54–56,223–225]
	Claudin	TGF-β3 (−), TNF (−), FSH/cAMP (−)	[43,44]
AJ-integral membrane	N-Cadherin	HGF (+), EGF (+), TGF-β (+/d), T (+), T↓ (+), IL-6 (−)	[30,66,78]
	E-Cadherin	TGF-β (−/d), T (+)	[52,66,68]
	Nectin-3	TGF-β3(−/d)	[52,68]
	Integrin-β1	TGF-β (+), T↓(+),	[30,226]
Adaptor	ZO-1	TGF-β (d), IL-4 (−), IL-13 (−), T↓(+)	[30,225]
	Afadin	TGF-β3(−/d)	[68]
	β-Catenin	TGF-β (d), T↓(+)	[30,139,227]
	α-Catenin	TGF-β (d), T↓(+)	[30,139,227]

T↓, suppression of intratesticular testosterone level with the use of testosterone (T) and estradiol implants; +, stimulation; −, inhibition. Protein distribution pattern was assessed by either immunofluorescent microscopy or immunohistochemistry using testicular cells cultured in vitro or seminiferous epithelium in vivo.

2.3.2.3. The integrin/laminin complex. The integrin/laminin protein complex has recently been identified at the apical ES which confers Sertoli–germ cell adhesion and provides a new platform regarding how these two cell types interact with each other and coordinate spermatogenesis [37,94]. Integrin-based protein complexes are usually found at the cell–matrix junctions, such as hemidesmosomes or focal adhesion, which further connects to the intermediate filament or actin bundles, with integrin also capable of serving as a cell receptor for the ECM [95,96]. Interestingly, the junctions between Sertoli and germ cells are not simple cell–cell junction types; rather, they are a hybrid of both cell–cell and cell–matrix junction types, probably to facilitate rapid junction turnover and germ cell migration during spermatogenesis [22]. Several recent reviews on the role of integrins and ECM in the testis are available, thus this information is not discussed herein [22,97].

3. Cytokines are key regulators of junction dynamics in the testis

Cytokines are regulatory peptides (usually ≤30 kDa in size) produced virtually by every nucleated cells in mammals and have pleiotropic actions on cell physiology as an autocrine or paracrine factor [98]. In the testis, Sertoli and germ cells produce a number of cytokines, including members of the TGF-β superfamily (e.g., TGF-βs, activins, inhibins), platelet-derived growth factor (PDGF), interleukins (e.g., IL-1, IL-6, IL-11), tumor necrosis factor (e.g., TNFα, Fas ligand), interferons (e.g., IFN-α, IFN-γ), fibroblast growth factor (FGF), nerve growth factor (NGF), and stem cell factor (or steel factor) (for reviews, see [1,2,11,99,100]) (see also Table 2). These cytokines likely mediate crosstalk between Sertoli and germ cells to facilitate germ cell movement across the seminiferous epithelium and other cellular events in the epithelium during the epithelial cycle such as germ cell differentiation. Herein, we critically evaluate two best studied cytokines, namely TNFα and TGF-β3, regarding their significance in spermatogenesis in the testis and briefly summarize the action of other cytokines.

3.1. TNF

TNF, also known as TNFα or cachectin, is synthesized as a 26 kDa type II transmembrane prepeptide (pro-TNF), which is subsequently activated by proteolytic cleavage to release the C-terminal 17 kDa mature protein by the TNF-converting enzyme (TACE). The mature protein is formed by aggregates creating a homotrimer that can bind to two types of receptors: TNFR1 and TNFR2 [101,102]. The major source of TNFα in mammalian body is immune cells such as macrophage and monocytes, but TNFα is also produced by other non-immune cells including astrocytes, keratinotytes, Sertoli cells and germ cells [57,101]. TNF signaling is mediated mainly through TNFR1, which has distinct domains that facilitate the recruitment of other intracellular adaptors to activate signaling pathways. The net result of such activation can modulate apoptosis, inflammation and cell proliferation [101,103]. These adaptors include TNFR1-associated death domain protein (TRADD) which can recruit Fas-associated death domain protein (FADD), TNF receptor associated factor-2 (TRAF-2), or receptor-interacting protein (RIP), to induce the caspase-mediated apoptosis, activate transcription factors (e.g., c-jun, c-fos, ATF-2) via MAPK (ERK, JNK and p38), or activate nuclear factor kappa B (NFκB) through inhibitor of NFκB kinase (IKK), respectively [101–103]. A TNFR1 scaffolding protein called TGFR-associated ubiquitous scaffolding and signaling protein (TRUSS) has recently been cloned and characterized [104]. The expression of TRUSS is enriched in heart, liver and testes, it is also known to interact with TRADD, TRAF-2 and IKK [104]. In addition to these complex signaling networks that can be activated down-

Table 2
Cytokines and their functions in the testis

Group	Cytokine	KO mice	Cellular expression	Function in the testis	References
TGF-β	TGF-β1	Perinatal/neonatal lethal	Sertoli, Leydig, germ, myoid cells	Testicular development	[109,112,119,123]
	TGF-β2	Perinatal lethal	Sertoli, Leydig and germ cells	Testicular development	
	TGF-β3	Perinatal lethal	Sertoli and germ cells	Junction dynamics	
	Activin/inhibin βA	Perinatal lethal	Sertoli and peritubular cells	Regulate FSH production, testicular development	[109,228]
	Activin/inhibin βB	Viable/reproductive abnormality (female)	Sertoli cells, germ cells		
	BMP-4	Embryonic lethal (−/−)/ lowered fecundity (+/−)	Pachytene spermatocytes, Sertoli cells (early postnatal)	Maintain spermatogenesis; spermatogonia differentiation	[109,229–231]
TNF	TNFα	Viable/fertile	Sertoli, germ cells	Repress steroidogenesis, disrupt TJ, inhibit GC apoptosis	[57,106–108,218,232]
	FasL	Viable	Spermatocytes/spermatids; Sertoli cell (?)	Induce apoptosis, preserve immune-privilege	[233–235]
	TRAIL	Viable/fertile	Germ, Leydig cells	GC apoptosis	[236,237]
Growth factors	EGF	Viable/fertile	Sertoli, germ cells	Maintain spermatogenesis; stimulate steroidogenesis;	[238–240]
	FGF4	Embryonic lethal	Sertoli cells	Enhance spermatogenesis	[241–243]
	HGF	Embryonic lethal	Spermatozoa, myoid cells	Initiate sperm motility, induce testicular cord formation	[244–246]
	MIF	Viable/fertile	Leydig cells	Leydig-Sertoli cell paracrine mediator/inhibit inhibin production	[247,248]
	SCF	Perinatal lethal (−/−), sterile (+/−)	Sertoli cells (c-Kit receptor on differentiating spermatogonia)	Spermtogenesis; Sertoli cell-spermatogonia adhesion (membrane bound form)	[11,249,250]
	VEGF (A)	Embryonic lethal (+/−)	Sertoli cells (receptor on germ cells), Leydig cells	Spermatogonial proliferation, spermiogenesis	[251–253]
Interleukin/interferon	IL-1α/-1β	Viable/lowered fecundity	Sertoli cells, spermatocytes, spermatids	Inhibit steroidogenesis, Regulate Sertoli secretion	[254,255]
	IFN-γ	Viable/fertile	Spermatogonia, interstitium	Inhibit steroidogenesis, stimulate FasL expression	[256,257]

Abbreviation: bone morphogenetic protein (BMP), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF, or scatter factor, SF), interferon (IFN), interleukin (IL), macrophage migration inhibitory factor (MIF), Stem cell factor (SCF, or Steel factor, SLF), transforming growth factor β (TGF-β), tumor necrosis factor α (TNF-α), tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL), vascular endothelial growth factor (VEGF).

stream of TNF, at least 19 ligands and more than 20 receptors have been identified in the TNF superfamily [103], which can mediate an array of physiological processes and diseases [103]. It has been known that TNF can disrupt TJ integrity in multiple epithelial cells. As aforementioned, TNF down-regulates occludin expression through its promoter activity [55] or reduces JAM-A distribution on the vascular endothelial cell surface [38]. TNF level is also elevated in Crohn's disease, a chronic granulomatous inflammatory disease that affects the gastrointestinal tract, which is manifested by impaired intestinal barrier function with leaky TJs [105]. With the exception of TNF and Fas ligand (FasL), the roles of other members of TNF superfamily in the testis remain elusive.

In the testis, TNFα has been shown to play a role in regulating germ cell apoptosis, junction remodeling and Leydig cell steroidogenesis [57,106,107]. For instance, it is known that TNFα represses the expression of steroidogenic-enzyme genes in Leydig cells through an activation of NFκB, which can in turn inhibit the transactivation of orphan nuclear receptors [106]. Intratesticular injection of TNF in normal and hypophysectomized rats has also demonstrated its suppressive effect on testosterone production in vivo [108]. Chronic infusion of TNF caused germ cell (in particular spermatocytes and spermatids) depletion from the epithelium, a loss of testis weight and a plunge in testosterone level [58]. It remains unknown regarding the mechanism(s) by which TNFα utilized to induce these changes, but this could involve a suppression of Leydig cells steroidogenesis, or an inhibition of Sertoli cell TJ protein production at the BTB, or via its direct effect on germ cells. Other recent studies have shown that TNFα can perturb the TJ-permeability barrier in cultured Sertoli cells dose-dependently and reversibly since the disrupted TJ-barrier can be resealed upon the removal of the cytokine [57]. This inhibitory effect of TNFα on Sertoli cell TJ function is likely mediated via an induced production of collagen α3(IV), matrix metalloprotease (MMP)-9 and tissue inhibitor of metalloprotease (TIMP)-1 which collectively affect the homeostasis of ECM, thereby altering the association of the Sertoli cell epithelium with the basement membrane and perturbing the TJ-barrier [57]. Also, TNFα can activate the integrin/integrin linked kinase (ILK)/glycogen synthase kinase (GSK) β-3/p130 Cas/JNK signaling pathway which also contribute to changes in the TJ-protein expression and/or distribution at the BTB [22,57,97].

3.2. TGF-β

The TGF-β superfamily comprises of TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), Müllerian-inhibiting substance (MIS) and others, totaling more than 35 members [109]. TGF-β superfamily proteins are crucial in the regulation of a variety of biological processes, including cell proliferation, differentiation, apoptosis, and tissue remodeling [110]. Some members, like activins and inhibins, were initially identified in the male gonad for their ability to regulate the pituitary follicle stimulating hormone (FSH) production [11]. MIS is known for its role in sexual differentiation causing the regression of the Müllerian ducts in the male [111]. The functions of TGF-β superfamily proteins in reproduction have been recently reviewed [109,112] hence we only focus on regulation of junction restructuring by TGF-βs herein, which is elaborated in Section 4. Table 2 summarizes other cytokines that are known regulators of junction dynamics.

3.3. Cytokines working in concert with other ECM proteins to regulate junction dynamics

Recent reviews have summarized how cytokines regulate the homeostasis of proteases and their inhibitors, and ECM proteins to coordinate spermatogenesis [2,22,97]. It is not at all surprising that these molecules are working in concert since their production, activation, and termination are all interdependent and connected. Their homeostasis and regulation are essential to almost all biological processes. In the testis, for instance, when the BTB is disrupted by cadmium, TGF-β3/p38 MAPK signaling is activated to down-regulate the steady-state of TJ and AJ protein levels that leads to the breakdown of both junctions and germ cell exfoliation [44,52,113]. Proteases (e.g., cathepsin L) and protease inhibitors (e.g., α_2-macroglobulin) are induced to coordinate the junction restructuring event [52]. Using a p38 MAPK inhibitor SB202190, the damage to the BTB and the plunge of TJ and AJ proteins induced by $CdCl_2$ can be delayed but it cannot prevent the overexpression of protease inhibitor α_2-MG [52]. Further study revealed that α_2-MG production is regulated by JNK signaling pathway in the testis, independent of the p38 MAPK pathway [114]. This yin and yang relation of protease and protease inhibitor regulation that utilizes distinct signaling pathways, and their connection with cytokines (e.g., TGF-β3) have illustrated that the testis is equipped with some delicate regulatory mechanisms to orchestrate junction restructuring at spermatogenesis.

4. TGF-β3 as a junction regulator—versatility realized through selectivity

4.1. Signaling conduits and versatile players in biological processes

TGF-βs (β1, β2 and β3) are key regulators in a plethora of biological processes (for reviews, see [110,115–120]). These cytokines, when activated by releasing from the latency-associated proteins (LAPs), can bind to their receptors—first to the type II receptor, TβRII, which then recruits the type I receptor, TβRI (or ALT5, activin-like kinase)—although TGF-β2 requires binding of the two receptors more or less at the same time and the assistance from the type III receptor, betaglycan. The binding of the cytokine to type I and type II receptors initiates a series of phosphorylation mediated activation—autophosphorylation of TβRII and TβRI phosphorylation by TβRII—and triggers consequent intracellular signaling events (the canonical Smad-mediated signalings and Smad-independent pathways). Despite their structural similarities and shared signaling mechanisms, the three TGF-βs are spatiotemporally expressed and play non-redundant roles, particularly under in vivo conditions. This in part is attributed to their unique promoter sequences [121]. For instance, in the mouse testis, expression of TGF-β1 and TGF-β2 are much higher in embryonic and early postnatal stages, and TGF-β3 becomes the highest expressed among the three isoforms in adulthood [122]. Similarly, in postnatal day 5 to day 60 rats, TGF-β1 and TGF-β2 expression are predominant in immature testes, which decrease at the onset of puberty; whereas TGF-β3 expression is most abundant at the pubertal stage, coinciding with the initiation of spermatogenesis [123]. These thus illustrate TGF-βs have unique roles in distinct phases of testicular development: TGF-β1 and TGF-β2 are important for the development while TGF-β3 takes the center stage during spermatogenesis. Herein, we summarize the TGF-β-mediated signaling conduits, focusing on their regulation of junction remodeling.

4.1.1. Smad-mediated signaling

The smad-mediated TGF-β signaling pathways have been extensively characterized and recently reviewed [110,116,124,125]. Among the 8 Smad proteins (Smad1–8), receptor-regulated R-Smad (Smad2 and Smad3), common-partner Co-Smad (Smad4) and inhibitory I-Smad (Smad 7) are involved in TGF-β/TβRII/TβRI signaling. However, many of these Smad proteins have not been subjected to rigorous investigation in the testis. In the testis, the expression of Smad2 and Smad3 are developmentally regulated and stage-specific: being more prominent in prepubertal than in sexually mature rats, and at the lowest levels at stages VII–VIII of the epithelial cycle in adult rats [126]. Expression of Smad3, 4, 6 and 7 are also detected in embryonic mouse testes [127]. It is not surprising that Smad proteins are highly expressed in younger animals since TGF-β superfamily members are essential for development. TGF-β1 and TGF-β2 may be more important in the testis at the early stages through Smad-mediated signaling pathways. Yet the regulation and maintenance of spermatogenesis by TGF-β3 in adult testes is likely mediated via Smad-independent signalings, such as TJ and BTB dynamics [68]. For instance, TGF-β3 activates ERK without activation of Smad2 and Smad3 in the Adjudin-induced germ cell loss model [68].

4.1.2. MAPK-mediated signaling

There are accumulating evidence in the literature regarding Smad-independent TGF-β signalings that regulate diverse biological function, which has recently been reviewed [115,116,118,128]. Amongst these, the best studied is the MAPK signalings [129–131]. For instance, TGF-β is capable of activating all three MAPK pathways [115,118,125]. In the testis, all three pathways have been implicated in the regulation of junction dynamics pertinent to spermatogenesis. First, JNK pathway is involved in TNF-α-induced TJ restructuring and α_2-MG regulation [57,114]. Second, ERK pathway can be activated via either integrin or TGF-β3, which can in turn regulate AJ dynamics [36,37,68]. Third, p38 MAPK is responsible for TGF-β3-activated TJ and AJ restructuring [52,113,132]. Nonetheless, little is known about the expression and distribution of MAPKs and their upstream kinases in the testis. ERK1/2 and p-ERK1/2 have been localized to the elongate spermatids at the apical ES/TBC site in the epithelium at stages VII–VIII [68,133], illustrating its role in spermiation. ERK1/2 is also detected at the basal compartment of the epithelium [133]. Indeed, when induced by Adjudin, p-ERK1/2 is activated at the site of apical ES in depleting elongate/elongating spermatids in tubules other than stages VII–VIII, probably facilitating germ cell exfoliation [68].

The complexity of TGF-β-mediated signaling pathways is manifested by the presence of multiple intracellular interacting points. Recent studies have identified different interacting proteins with TGF-β receptors, illustrating these proteins may play a role in selecting the downstream signaling events. For example, occludin is known to associate with TβRI and as such, TGF-β can efficiently regulate TJ disruption during epithelial-mesenchymal transition (EMT) [134]. Indeed, the proximity of TGF-β receptors with TJ proteins has created an efficient regulatory mechanism where TGF-β-induced TJ dissolution is mediated through the cell polarity complex. Upon activation by TGF-β, TβRII is recruited to the TβRI/occludin/Par6 complex, thereby phosphorylating Par6, this in turn stimulates Par6 which binds to Smurf1 (an E3 ubiquitin ligase), and causing degradation of RhoA that leads to TJ disassembly [135,136]. Although it has not yet been confirmed for TβRII, proteins that associate with type II receptor of BMP have recently been identified, which include MAPK, PKC, and cytoskeleton tubulin β5 [137]. These proteins associate not only with the kinase domain of the receptor but also its C-terminus [137], illustrating receptors of the TGF-β family proteins can affect junction dynamics via protein-protein interactions with junction protein complexes.

4.1.3. TGF-βs regulate junction restructuring

TGF-βs regulate junction dynamics in various cell types. For instance, TGF-β1 can perturb the permeability of the blood–retinal barrier via a stimulation of MMP-9 production [138]. TGF-β1 also perturbs the TJ-permeability barrier in pulmonary endothelial monolayers by inducing AJ proteins to move away from the cell–cell contact site, possibly via a myosin light chain kinase mediated mechanism [139]. TGF-β1 and Ras can also work synergistically to promote cell invasiveness in intestinal epithelial cells by down-regulating E-cadherin expression and subcellular redistribution of β-catenin [140]. In addition, TGF-β1 can induce AJ disruption in renal proximal tubular epithelial cells, which cannot be reproduced by transient overexpression of Smad2/4 or Smad3/4 [141], illustrating this is an Smad-independent signaling event. On the other hand, a blockage of TGF-β signaling by treatment of a TGF-β receptor kinase inhibitor up-regulates TJ protein production (e.g., claudin-5) in embryonic stem cell-derived endothelial cells [142]. Interestingly, in almost all of these epithelial/endothelial cells, a disruption of either TJ or AJ can affect the integrity of the other junction type following an induction by TGF-βs. Yet the functional inter-relationship of AJ and TJ in the seminiferous epithelium is significantly different from all other epithelia and endothelia. For instance, TGF-β3 (and also TGF-β2 in vitro) can disrupt the Sertoli–Sertoli TJ-barrier by down-regulating TJ proteins (e.g., occludin) via p38 MAPK signaling pathway and this effect is indeed confirmed using an in vivo model to study the BTB dynamics [44,113,132] (see Fig. 2). Analogous to other epithelia and endothelia, a breakdown of TJ can indeed affect the integrity of AJ, resulting in a loss of Sertoli–germ cell adhesion [52]. However, a disruption of AJ between Sertoli–germ and Sertoli–Sertoli cells seems to reinforce the TJ at the BTB instead, let alone its disruption, in the Adjudin- and intratesticular testosterone suppression-induced germ cell loss models [2,30]. Recent studies have shown that TGF-β3 can exert its effects on AJ integrity via a signaling pathway different from the one that regulates TJ dynamics in the testis [68], so that Sertoli–germ cell AJ can undergo restructuring without perturbing the BTB integrity (Fig. 2). This unique relation of AJ and TJ in the seminiferous epithelium may be a physiological requirement for the testis to facilitate germ cell migration (i.e., AJ restructuring) while maintaining TJ integrity. This concept will be revisited and discussed in detail in Section 5.

4.2. Signaling regulation and selectivity

4.2.1. Multilayers of signal modulation

Regulation of TGF-β-mediated signalings occurs at multiple levels: ligand production and activation, ligand–receptor coupling, intracellular signal pathway selection, nucleocytoplasmic shuttling of transcription factors, an interaction of multiple transcription factors that finally determines the activation or repression of gene expression, and signal termination [110,143–146]. Less is known regarding how the expression of TGF-βs is regulated. The promoter sequences of human TGF-βs have been characterized. For instance, TGF-β1 is mostly regulated by AP-1 site lacking TATA box, whereas TGF-β2 and TGF-β3 are regulated by AP-2 site and cAMP-responsive elements, containing TATA box [121], and the most potent activator of TGF-β1 expression known thus far is the cytokine itself [147]. It has been shown that JNK suppresses the autocrine expression of TGF-β1 in fibroblasts [148]. A recent in vivo study in the testis has shown that JNK signaling is required for the production of α_2-MG in the seminiferous epithelium, which tethers TGF-β3 and antagonizes the cytokine [114]. These results thus illustrate the TGF-β action is regulated at multiple levels and can induce diversified biological responses. Upon secretion, TGF-βs are tightly but non-covalently bound to LAPs, which are further tethered to latent transforming growth factor-β binding proteins (LTBPs) via covalent bonds [149]. LTBP can covalently bind to ECM, enabling cytokines to be retained in the matrix and creates a reservoir [149]. This biologically inactive cytokine pool can be activated by low pH, protease (e.g., plasmin, MMP-2 and MMP-9), thrombospondin-1 (TSP-1), integrin-αvβ6 or -αvβ8 [145,146,149,150]. At least one LTBP called LTBP-1L (long form) is highly expressed in testes [150]. MMP-2 and MMP-9 are also found in the testis [97]. Other antagonists of TGF-βs include α_2-MG and decorin, which can ‘lock’ the ligand and prevent its binding with receptors, and endoglin, which binds to TβRII-associated TGF-β1 or -β3 and attenuates TβRI mediated signaling [110,151,152]. After TGF-β binds to its receptors, signaling is triggered but can be directed to a distinctive pathway, and can sometimes activate multiple pathways. Because of such diversified signaling capacity, a mechanism must be in place to choose the needed downstream signaling pathway. It is likely that adaptor proteins play the decision-making role. For instance, activation of Smad2/3 is facilitated by the adaptor SARA. Yet the detail of this selection still remains elusive. To transmit the signaling to the corresponding genes for their transcriptional induction, activated transcription factors (e.g., Smad2 and Smad3) must enter the nucleus. As such, there is constant nucleocytoplasmic shuttling of the R-Smads between active (phosphorylated) and inactive (dephosphorylated) status to keep sensing the signals at real-time [110,144]. The cell-specific and non-specific transcription factors/coactivator/co-repressors can determine the final gene expression outcome in a particular cell type at the end of TGF-β activation [110,116]. Receptor internalization and degradation, Smad shuttling and ubiquitination, and expression feedback can all contribute to the signal termination [118].

4.2.2. Adaptors as molecular switches for TGF-β signaling in the testis

It is of interest to note that in the testis, the TGF-β3-activated signaling can have distinctive effects on the junction restructuring. When p38 MAPK is activated by TGF-β3, the BTB in the seminiferous epithelium is disrupted concomitant with Sertoli–germ cell AJ disassembly [52] (Fig. 2). In contrast, when ERK1/2 is activated by TGF-β3, only AJs are affected without affecting the BTB integrity [68] (Fig. 2). Indeed, a blockade of the TGF-β3-mediated signaling by using an antagonist (e.g., TβRII/Fc conjugate) can prevent the activation of ERK1/2 and significantly delay the Adjudin-induced germ cell loss from

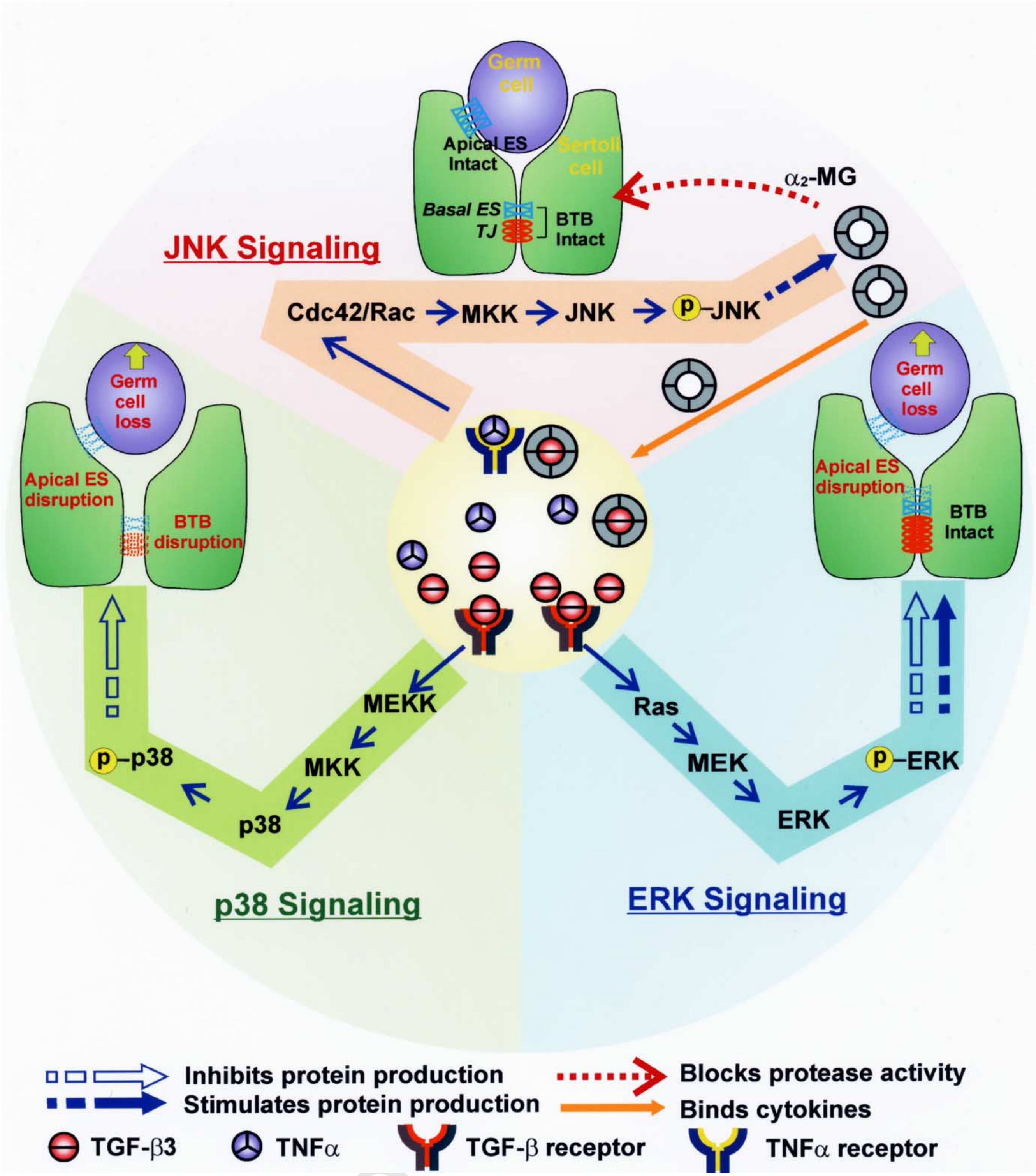

Fig. 2. A schematic illustration of how cytokines (e.g., TGF-β3, TNFα) can regulate junction dynamics in the testis via their effects on the steady-state levels of proteins (e.g., TJ- and AJ-proteins, protease inhibitors such as α_2-MG) at the BTB and apical ES. This model was prepared based on recent studies from this laboratory using different animal models as reviewed herein. In brief, cytokines released from either Sertoli or germ cells can activate at least three different signaling pathways upon their binding to receptors. For instance, TGF-β3 can activate p38 MAPK signaling pathway to down-regulate both TJ and AJ proteins, resulting in the disruption of the BTB and Sertoli–germ cell adhesion function, eventually leading to germ cell loss from the epithelium (green sector), which was identified in studies using the cadmium model [52,113,132]. This also illustrates that when TGF-β3 utilizes the p38 MAPK pathway for its signaling function, it can perturb both the BTB and apical ES integrity. When rats were treated with Adjudin, or testosterone/estradiol implants to reduce intratesticular androgen level, the testis responds to these treatments with an induction of TGF-β3 that can activate only the ERK signaling pathway to compromise Sertoli–germ cell adhesion function by lowering the steady-state protein levels at the apical ES or weakening protein-protein interactions at this site via changes in the phosphorylation status of adaptors (e.g., β-catenin); this in turn leads to the loss of germ cells from the epithelium, and this event does not affect the BTB

the epithelium [68]. By blocking these two MAPK signaling pathways using kinase inhibitors can also rescue the epithelium from the disruptive effects of $CdCl_2$ and Adjudin on the BTB and Sertoli–germ cell AJ, respectively [52,68,113]. As such, TGF-β3 serves as a key regulator that decides whether BTB is affected or not. It is tempting to speculate that this TGF-β-activated MAPK signaling cascade requires the recruitment of adaptors to the site which can in turn shuttle to the correct signaling pathway downstream. Recent studies have shown that TGF-β-induced p38 MAPK activation is mediated through a protein scaffold complex XIAP (X-linked inhibitor of apoptosis)/TAB1 (TAK binding protein)/TAK1 (TGF-β-activated kinase), in which adaptor XIAP may link adaptor TAB1 and MAPKKK TAK1 to the TβRI. TβR1 can activate TAK1, which further activates either MKK3/6 or MKK4 that in turn activates p38 MAPK or JNK, respectively [118]. Besides, MAPKK-independent autophosphorylation of p38 is also possible, which is TAB1 dependent [153]. On the other hand, TGF-β can activate Ras, which further activates ERK signaling pathway and regulates various cellular processes including junction dynamics [68]. TGF-β-induced ERK activation also requires the adaptor CD2-associated protein (CD2AP). When this adaptor is not involved, p38 MAPK is preferentially activated instead. In its presence, TGF-β activates both the PI 3-kinase/Akt and the Ras/ERK pathways [154]. Interestingly, CD2AP is not involved in PI 3-kinase/Akt activation by EGF and insulin, nor in the activation of Smad2 by TGF-β, suggesting it plays a role in TGF-β-activated, Smad-independent signaling [155]. The association between TGF-β receptors and CD2AP is further supported by the evidence that both are present in lipid rafts [156,157]. Thus CD2AP can serve as a molecular switch to determine the downstream signaling direction of TGF-β. CD2AP belongs to a family of ubiquitously expressed adaptors containing three Src-homology 3 (SH3) domains, a proline-rich region and a coiled-coil domain [158] and is expressed in human testes [159]. The SH3 domain mediates interaction with the p85 subunit of PI 3-kinase and the proline-rich region mediates association with p130 Cas and Src family kinases (for a review, see [158]). p130 Cas and Src are components of a signaling machinery connecting FAK, paxillin, ERK and myosin light chain kinase (MLCK), which, in turn regulate cell adhesion during cell migration process [160]. Interestingly, virtually all of these proteins have recently been found in the testis and they are likely involved in junction dynamics during spermatogenesis (for reviews, see [2,22,97]). It is likely that CD2AP is a crucial adaptor of TGF-β mediated and integrin/FAK mediated signaling events in the testis. Although much of the information on CD2AP derives from studies in the kidney, the testis may employ this molecular switch to select the downstream signaling pathways to be activated by TGF-β3, affecting either AJ alone or TJ and basal ES at the BTB. This should be vigorously validated in future studies.

5. What lessons we learn from the testis as a model to study junction restructuring?

As we have discussed above, the testis is an intriguing organ where extensive junction restructuring occurs in the seminiferous epithelium at each stage of the epithelial cycle. Recent studies aiming to delineate the mechanisms that regulate the junction restructuring events in the testis have yielded some crucial information, which is likely applicable to general cellular physiology as a whole. Herein, we summarize several in vivo models that have been established and used in recent studies (see Table 3). We only highlight some of the latest development using these models and readers are encouraged to refer to several recent reviews [1,2,9,75,161,162].

5.1. Adjudin model

Formerly called AF-2364 [1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide], Adjudin is a molecule that selectively induces adherens junction disruption. It is a well studied potential male contraceptive derived from indazole-3-carboxylic acid [163,164]. It is also one of the best studied compounds that induce germ cell sloughing in the testis. Adjudin apparently exerts its effects on the Sertoli–germ cell adhesion unit to induce a loss of AJ function by triggering a couple of signaling events, including a surge of the ES-associated signaling molecule testin, an induction of integrin- and cadherin-initiated pathways, as well as TGF-β3 activation [37,66–68,74,163,165–167]. Moreover, the ES-based AJs are compromised due to a loss of protein-protein association in the N-cadherin/β-catenin and nectin/afadin protein complexes, which is likely the result of a coordinated regulation by protein and lipid kinases and phosphatases, proteases and protease inhibitors [35,67,68,74,94]. These signalings are triggered within a few hours after adult male rats are treated with a single or multiple doses of Adjudin at

integrity (blue sector) [2,68]. This thus suggests that TGF-β3 can limit its action at the apical ES without compromising the BTB when the ERK signaling pathway is being utilized. Using the cadmium model, it is presently known that JNK is activated during the cadmium-induced BTB damage. This induces the production of α_2-MG, which either bind to the free cytokines, limiting their biological action and/or blocking protease activity to limit the BTB damage. Since it is known that by blocking the production of α_2-MG, it can worsen the damaging effect of cadmium on testicular junctions (red sector), illustrating this JNK-α_2-MG pathway is crucial to maintain the normal physiology in the seminiferous epithelium [57,114]. This pathway is likely utilized by TNFα to regulate the steady-state protein level of α_2-MG. The coordinated action of these three interacting signaling pathways that are intriguingly regulated by cytokines (e.g., TGF-β3 and TNFα) is crucial to maintain the integrity of the seminiferous epithelium during spermatogenesis, permitting selective disruption of either TJ, AJ, or TJ and AJ. As reviewed herein, it is likely that adaptors play a crucial role upstream to select which signaling pathway should be activated by these cytokines, which in turn determines if either BTB, apical ES, or both BTB and apical ES should be compromised during spermatogenesis.

Table 3
Chemicals that target the testis and can potentially serve as models to study junction dynamics

Affectedjunction types	Chemical	Classification	Target junction types in the testis and manifestations	References
TJ/AJ	Cadmium	Heavy metal	BTB/ES disruption; germ cell loss/apoptosis, irreversible	[52,114,178,179,200]
TJ	Cisplatin	Chemotherapeutic drug	BTB disruption; azoospermia, irreversible	[177,258,259]
TJ	Glycerol	1,2,3-Propanetriol	BTB disruption; germ cell loss, irreversible	[260,261]
TJ	Occludin peptide	22-a.a. from 2nd extracellular loop of occludin	BTB disruption; germ cell loss, reversible	[262]
AJ/TJ	Gossypol	Extract from cotton seed oil	ES/AJ/prevent BTB formation in neonatal animal; germ cell loss; irreversible in neonatal animal	[177,263–265]
AJ	Adjudin (AF-2364)	Indazole-3-carboxylic acid analog	ES/AJ disruption; germ cell loss, reversible	[163,164]
AJ	AF-2785		ES/AJ disruption; germ cell loss, reversible	[163,164]
AJ	Lonidamine (AF-1890)		ES/AJ disruption; germ cell loss; irreversible in selected subjects	[266]
AJ	Testosterone/estrogen implants	Steroid hormone	ES/AJ disruption; germ cell loss, reversible	[30,33,35,36,267,268]
AJ	Vinclozolin	Fungicide/antiandrogen	ES/AJ (?); germ cell apoptosis	[180,191,269,270]
AJ	Phthalate	Widely used as a plasticizer and in cosmetics/antiandrogen	Basal and apical ES disruption; seminiferous tubule atrophy, germ cell loss	[180,181,183,186]
AJ	Bisphenol A	Plastics/estrogenic	Apical ES disruption; abnormal spermatids, acrosomal defects	[177,182,198,271–273]

40–50 mg/kg b.w. either via i.p. or by gavage. Thereafter, morphological changes (i.e., germ cell depletion) are typically seen by 6–8 h [168]. The effect of Adjudin is limited to AJs, since the BTB remains intact in Adjudin treated rats. Furthermore, spermatogonia cell population apparently is unaffected [2]. Its antifertility effects are reversible, since the voided tubules treated with Adjudin can become repopulated with germ cells, making them almost indistinguishable from normal testes [164]. Studies from Adjudin treated rat testes have revealed some regulatory mechanisms that affect Sertoli–germ cell adhesion function pertinent to spermatogenesis. For instance, the integrin/FAK signaling is activated during Adjudin-induced germ cell loss from the epithelium [37]. This information has recently been validated and expanded using an androgen suppression-induced germ cell loss model in which rats received androgen and estrogen implants to suppress the intratesticular androgen level thereby perturbing Sertoli–germ cell apical ES function [36,37]. More important, TGF-β3 is also induced in androgen-suppressed rat testes, similar to the Adjudin model ([68] and unpublished observations), illustrating the involvement of cytokines in cell adhesion function. It is possible that the migration of germ cells across the seminiferous epithelium during spermatogenesis is controlled by several independent signaling pathways. When an agent activates these signalings, though the initial responses are different for different agents, the net outcome (i.e., alteration in Sertoli–germ cell adhesion function and the subsequent germ cell sloughing) is similar. Indeed, the signaling events in the rat testis identified using the Adjudin model have shown that this organ is utilizing the junction restructuring events usually restricted cell-ECM interface to regulate cell adhesion, migration, tissue remodeling and development, and tumor cell metastasis [115,169,170], illustrating the cell–cell anchoring junction in the testis is indeed a hybrid cell–cell and cell–matrix junction type [22].

5.2. Cadmium model

Cd is a heavy metal and an environmental pollutant that is widely used in industry. It poses significant threat to human health and is classified as an endocrine disruptor [171–173]. It adversely affects a number of organs including the testis, kidney, lung, liver, pancreas and placenta [171,173]. The molecular mechanisms of action of cadmium toxicity are rather diverse which include: (i) binding to estrogen receptors, mimicking estrogen in the uterus and mammary gland [174]; (ii) disrupting the cadherin-based cell–cell adhesion [172]; (iii) inhibiting the DNA mismatch repair [175]; and (iv) disrupting endothelial and blood-testis barriers [52]. The testis is very sensitive to Cd exposure and the Cd-induced testicular effects (e.g., necrosis) is common across all animal species [176]. The antifertility effect of Cd has been known for decades. A recent study has identified a metal transporter of Cd (ZIP8, ZRT-, IRT-like protein 8) that is highly expressed in Sertoli cells [176], which likely explains, at least in part, why this cell type is sensitive to Cd-induced damages in the testis. Indeed, the junctional proteins are the early targets of a panel of toxicants, including Cd, in Sertoli cells cultured in vitro [177]. At a relative low dosage of Cd (e.g., 0.1–1 μM), it can reversibly perturb the Sertoli cell TJ-barrier in vitro when testosterone and FSH are present in the media [178]. Intraperitoneal administration of cadmium (1–3 mg/kg b.w.) to adult rats can irreversibly damage the BTB, which has been used as a model to study

BTB dynamics in the testis [52,113,114,179]. Apparently, when absorbed by Sertoli cells, Cd targets the microfilament, causing a disorganization of actin bundles [179]. Furthermore, Cd induces the dissolution of TJ proteins (e.g., occludin) from the seminiferous epithelium, and down-regulates AJ-proteins (e.g., cadherin, nectin) to induce a secondary disruption of basal ES, leading to germ cell sloughing [52]. Using this model, it has been shown that the TGF-β3/MEKK/p38 MAPK mediated signaling pathway is a putative mechanism that regulates TJ dynamics at the BTB in vivo, and a disruption of this pathway using specific inhibitors can indeed significantly delay the Cd damage to the BTB as well as the subsequent germ cell loss from the epithelium [44,113,132].

5.3. Possible in vivo models to study junction dynamics in the testis

Recent studies have illustrated a number of chemicals that can affect testicular junctions, which may be developed into useful in vivo models. Many of these molecules are endocrine disruptors, which include phthalate, bisphenol A, vinclozolin and others [180–183]. Because of their widespread distribution in the environment and potential health hazards (e.g., reproductive organs), these compounds have attracted great attention of research, particularly on their effects to the reproductive organs (e.g., testes). They affect the endocrine system either by acting as antiandrogens (e.g., phthalate, vinclozolin) or estrogens (e.g., bisphenol A) [180–183].

Phthalate and vinclozolin are compounds that can antagonize androgens. However, they exert these effects via different mechanisms: phthalate affects androgen synthesis [183] whereas the metabolites of vinclozolin are antagonists of androgen receptors [184,185]. Phthalate is found in cosmetic products (e.g., nail polishes, perfumes, hair sprays) and is widely used as a plasticizer, which can be non-covalently bound to the matrix and thereby slowly releases to the environment, and can be inhaled or adsorbed dermally [181,183]. Its toxic effects in male neonatal animals include hypospadias, reduced anogenital distance, vaginal pouch, some of which (e.g., hypospadias) are detected in humans [180,181,183,186]. When adults rats were treated with a single dose of di-*n*-pentyl phthalate (DPP) (2.2 g/kg b.w.), Sertoli cell junctions displayed abnormalities with disrupted basal ES, and apical ES was either absent or badly disorganized [187]. Interestingly, the disrupted basal ES between apposing Sertoli cells were reformed by 48 h after DPP treatment [187], illustrating this is a potentially useful model to study basal ES dynamics if adequately characterized. Furthermore, in DPP-treated prepubertal rats, extensive vacuolation occurs in Sertoli cells, to be followed by sloughing of germinal cells [188]. Apparently the observed effects of phthalate on germ cell loss are mediated via disruption of Sertoli-Sertoli and Sertoli–germ cell adhesion function.

Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione] is a fungicide that is widely used in farming industry. When adsorbed by humans or rodents, vinclozolin is metabolized to M1 (2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid) and M2 (3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide), which can bind to androgen receptors, antagonizing androgen function in vivo [184,185]. Besides its disruptive effects on reproductive organs (e.g., hypospadias, reduced anogenital distance) in male rats when exposed to vinclozolin in utero, it is neurotoxic and is an endocrine disruptor [189]. In the testis, vinclozolin can induce Leydig cell hypertrophy, reduce testis weight as a result of germ cell loss, and subsequently impair sperm production [190–192]. The direct structural damage of junctions at the Sertoli–Sertoli and Sertoli–germ cell interface following vinclozolin treatment remains to be examined.

Bisphenol A is a xenoestrogen although its bioactivity is 1000–1500-fold lower than 17β-estradiol. An ultrastructural examination of adult rat and mouse testes after treatment with bisphenol A has revealed that the apical ES was absent or badly damaged versus control animals; but, interestingly, the basal ES and BTB were not affected [182]. This information has also strengthened the notion that AJ disruption in the seminiferous epithelium can be restricted to the ES site without perturbing the TJ-barrier function at the BTB [2,30] in contrast to other epithelia where a disruption of AJ can lead to a secondary damage of the TJ-barrier function and vice versa [193–197]. The disruptive effects of bisphenol A and estrogens (17B-estradiol and β-estradiol-3-benzoate) on apical ES (but not basal ES and BTB, which remained intact) was also detected in maturating rats and mice that had been exposed to bisphenol A at neonatal [198]. However, these effects were not found when rats were fully mature [198]. In short, one of the target structures of this endocrine disruptor is the apical ES.

5.4. Why is the testis a vulnerable target of environmental toxicants? A lesson to learn from the testis

Studies on different environmental toxicants have unequivocally demonstrated that the testis is extremely vulnerable to these toxicants (for a review, see [199]). When exposed to these chemicals, Sertoli-Sertoli and Sertoli–germ cell junctions are the early targets and their subsequent dissolution is likely the result of down-regulation of junction proteins or changes in protein-protein association of the junction protein complexes [177]. Indeed, recent studies have shown that when adult rats were exposed to cadmium chloride, the BTB damage had occurred at least 24 h before the TJ-barrier of the microvessel in the interstitium [114,200], indicating the BTB is more sensitive than the endothelial TJ-barrier in microvessels to cadmium toxicity. Subsequent analyses by immunohistochemistry and fluorescent microscopy using these rats have conclusively demonstrated a significant loss of TJ- and AJ-integral proteins from the BTB site, consistent with results of immunoblot analyses [52,114]. Furthermore, a loss of protein-protein interactions of the AJ integral membrane proteins and their adaptors namely cadherin-catenin and nectin-afadin was also detected when rats were exposed to Adjudin [68], a chemical known to induce germ cell loss from the seminiferous epithelium without disrupting the TJ-barrier at the BTB (for reviews, see [1,2,9]). Taking collectively, these data have clearly illustrated the vulnerability of the testis to environmental toxicants (e.g., cadmium) and that the proteins at the TJ and AJ sites are some of the primary targets of these toxicants. While the precise mechanism underlying such vulnerability is not fully understood, recent studies have shed new lights on this issue, which also highlights a unique opportunity to use these toxicant-induced BTB or AJ damage to the testis as novel models to study BTB dynamics, AJ restructuring pertinent to spermatogenesis, and their regulation. Furthermore, these studies can plausibly provide new insights in developing preventive measures to antagonize these toxicants.

First, Sertoli cells are secretory cells that actively provide virtually all the necessary nutrients for germ cell development behind the BTB including metal transporters, such as transferrin, ceruoplasmin, and metallothioneins (MTs). MTs are small Mr proteins having high affinities for heavy metal ions including cadmium, zinc, copper and mercury. MTs are produced in virtually all mammalian tissues in response to metal ions exposure, which can detoxify heavy metals, such as cadmium (for reviews, see [201,202–205]). MTs are also important to maintain the homeostasis of essential trace elements, such as zinc and copper and are scavengers of free radicals [202,204] and protect cells from the cytotoxic effects of cadmium [206]. In the rat testis, MTs, such as MT1 and MT2, have been identified and isolated [207,208]. Recent studies have found a novel testis-specific MT-like protein called tesmin which is specifically expressed by spermatogenic cells [209]. MT1 and MT2 are products of Sertoli and germ cells, which are significantly induced after cadmium exposure [210]. Yet the production of MTs by Sertoli and germ cells are significantly lower when compared to hepatocytes in vitro in response to cadmium exposure [210]. Indeed, the quiescence of MT expression in the ventral prostate and the testis is the possible cause of their susceptibility to cadmium cytotoxicity and carcinogenicity [204,211–213].

Second, recent studies on the effects of cadmium on different cell lines, including MDCK, LLC-PK1, and Caco-2 cells, have shown that its primary target is E-cadherin (for a review, see [214]). For instance, cells that were exposed to cadmium were found to have their E-cadherin moving away from the cell–cell interface and became diffusely localized in the cytoplasm. It was postulated that cadmium may be competing to the binding of calcium to the E-cadherin, thereby perturbing the AJ function [214–217]. If this is the case, cadmium (and possibly other environmental toxicants) must first gain access to AJ to disrupt E-cadherin. In all other epithelia found in mammals, AJ is physically located behind the TJ since the TJ-barrier is located to the apical portion of the cell epithelium, and behind TJ lies desmosomes, which collectively known as the junctional complex. Behind the junctional complex are the gap junctions to be followed by the cell–matrix adhesion complex. As such, the TJ would seal most of the environmental toxicants off the epithelium in virtually all organs. Yet in the testis, TJ coexists with AJ and desmosome-like junctions at the BTB, which collectively lies adjacent to the basement membrane (a modified form of ECM, for a review, see [12]), closest to the interstitium. Thus, toxicants (e.g., cadmium) diffuses from the microvessels will have immediate access to the E-cadherin in the AJ (which is the cellular target of cadmium) at the BTB because there is no TJ-barrier that seals off cadmium. This, in turn, disrupts AJ, inducing germ cell loss from the epithelium as manifested by germ cell sloughing in many of these animal models using environmental toxicants. It is of interest to note that recent studies have begun to shed light on the physiological significance of such coexisting TJ and AJ at the BTB in relation to spermatogenesis. For instance, it is well understood that spermatogenesis is associated with extensive restructuring of Sertoli–Sertoli and Sertoli–germ cell interface because of the constant reshaping of germ cell shapes as a result of differentiation and germ cell movement from the basal to the adluminal compartment. If such AJ restructuring leads to TJ-barrier disruption as it is the case in other epithelia [193,195,197], the BTB integrity cannot be maintained, and haploid germ cell antigens cannot be sequestered from the host immune system; and such a disruption, even transiently, of the immunological barrier is detrimental to spermatogenesis. Thus, the fact that the BTB is constituted by co-existing AJ and TJ is to ensure such transient disruption of TJ during AJ restructuring in the seminiferous epithelium does not occur. Recent studies have shown that a signal that induces AJ disruption [e.g., via treatment of rats with Adjudin to induce extensive AJ restructuring that leads to germ cell loss from the epithelium, or a decline in endogenous intratesticular T level using androgen/estradiol transdermal implants] can lead to a surge in the production of both AJ (e.g., cadherins, catenins) and TJ (e.g., occludin, ZO-1) proteins [30,35,68] (Fig. 2). The increased TJ proteins are being used to reinforce the TJ-barrier integrity at the BTB at the time of extensive AJ restructuring. While the levels of AJ proteins are also induced, germ cells can still be dissociated from Sertoli cells because the AJ-integral membrane protein-AJ adaptor (e.g., the N-cadherin-β-catenin protein complex) association is found to be weakened via an increase in tyrosine phosphorylation of β-catenin [30,35,68]. These findings are significant because it depicts the presence of a novel mechanism utilized by the testis to ensure TJ-barrier integrity while permitting AJ restructuring within a microenvironment such as the seminiferous epithelium. Fig. 2 is a schematic drawing that illustrates this novel mechanism of increasing AJ and TJ proteins, which is likely regulated by cytokines [e.g., TGF-β3, TNF-α released from either Sertoli or germ cells [57,113,123,218]] via the ERK signaling pathway that maintains the BTB integrity while permitting AJ restructuring, facilitating germ cell movement across the seminiferous epithelium. However, this same mechanism that is physiologically necessary to facilitate germ cell movement while maintaining BTB integrity during spermatogenesis also makes the BTB extremely vulnerable to environmental toxicants because of the unusual exposure of AJ structural proteins (e.g., E-cadherin) to the toxicants (e.g., cadmium).

6. Conclusion and future perspectives

In all animal species, cell migration and junction remodeling are naturally occurring processes. For instance, there are three types of signals that control different aspects of *Drosophila* border cell migration: a global steroid-hormone signal to determine the timing, a highly localized cytokine signal to induce migration, and a growth factor to guide cells to their destination (for a review, see [219]). In the testis, FSH released from the pituitary and testosterone from Leydig cells may serve as the global regulatory factors. Cytokines that function as either paracrines or autocrines can in turn regulate localized signaling and processes. Several theories of germ cell movement during spermatogenesis have been proposed, and recently, we have put forth a junction restructuring theory in which cytokines, protease/protease inhibitors, cytoskeleton regulators and junctional complex proteins are all coordinated to facilitate germ cell movement in the epithelium [2]. Stem cell research has also offered new insights on Sertoli–germ cell interactions and may facilitate the research regarding the local regulatory function of cytokines in this event. When rat spermatogonia are transplanted into recipient mouse testes, the rat stem cells develop according to their only timing (∼7 week instead of ∼5 week), irrespective to the surrounding mouse spermatogenesis milieu [220]. It seems that this internal preprogrammed rhythm autonomously determines the fate of rat spermatogonia differentiation, and creates a suitable localized environment through dialogues with mouse Sertoli cells, probably via cytokines for crosstalk. This intrinsic preprogrammed timing may be controlled or executed by homeobox genes. A homeobox gene cluster Rhox (reproductive homeobox on the X chromosome) has recently been identified in mice [221]. The 12 Rhox genes are expressed mostly in reproductive organs (placenta, ovary, testis and epididymis), arranged into three subclusters and manifested temporal and quantitative colinearity in expression patterns [221]. In the testis, the majority of Rhox genes are primarily expressed in Sertoli cells and androgen responsive [221]. During the first wave of spermatogenesis, the timing of Rhox genes expression corresponds to the specific phases of germ cell differentiation. Hence these transcription factors may direct the expression of an array of proteins required for germ cell development, and may also define the corresponding timing of epithelial cycle and length of spermatogenesis in rodents [221].

Several approaches can be used in future studies to aid the understanding of Sertoli–germ cell crosstalk and junction restructuring. First, development of testis-specific knockout mice against crucial proteins pertinent to junction restructuring and spermatogenesis to identify the function of these proteins in the testis. For many cytokines (e.g., TGF-βs), their deletion can lead to lethality of the null mice. As such, their roles in spermatogenesis at adulthood cannot be examined. In the rat, the first wave of spermiation occurs only by 30–40 days of age. Recently conditional knockout technique has allowed investigators to elucidate protein function in a tissue- and time-specific manner in testis using specific Sertoli cell KOs, such as androgen receptor [222]. The generation of testis-specific KOs (e.g., TGF-β3) will help define the roles of these cytokines in junction dynamics at spermatogenesis. Second, germline stem cell transplantation with traceable markers to follow germ cell differentiation as well as junction remodeling during spermatogenesis can assist the study of cell–cell interactions pertinent to germ cell movement. For instance, when spermatogonia are transplanted into the recipient testis, they can migrate to the basal niche and initiate spermatogenesis in the preprogrammed cycle independent of the host environment. This migration must traverse the BTB, differing from gonocyte migration in the tubule when BTB has not yet formed. Third, using microarray technique to identify the expression profiles of various cytokines, proteases and protease inhibitors, junctional proteins, adaptors and transcription factors in staged tubules and in testes obtained from selected in vivo models. This approach can also pinpoint the leading and supporting biological factors pertinent to spermatogenesis. It is hopeful that using these approaches, a better understanding of spermatogenesis can emerge, which should be helpful for various applications such as treating male infertility or for contraception.

Acknowledgements

Supported in part by grants from the National Institutes of Health (NICHD, 5U01 HD045908 to CYC; 5U54 HD029990, Project 3 to CYC), and the CONRAD Program (CICCR, C1G 01-72 to CYC, CIG 01-74 to DDM).

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